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In vivo footprinting was utilized to examine the estrogen-responsive pS2 gene in MCF-7 human breast cancer cells. Estrogen treatment resulted in an extensive region of protection within and adjacent to the pS2 estrogen response element (ERE). The antiestrogens 4-hydroxytamoxifen and ICI 182,780 each produced distinct protection patterns of the pS2 ERE. The TATA and CAAT sequences were protected and flanked by hypersensitive sites in the presence and absence of hormone in MCF-7 cells but not in estrogen receptor (ER)-negative MDA MB 231 cells. To aid in future identification of proteins bound to these elements, *in vitro* footprinting experiments were performed utilizing purified ER and nuclear extracts from estrogen-treated MCF-7 cells. Results of *in vitro* DNase I footprinting experiments paralleled the protection patterns of *in vivo* footprinting experiments. The CAAT box was minimally protected with hypersensitive sites surrounding both the TATA and CAAT boxes. Our studies suggest that proteins present in nuclear extracts from estrogen-treated MCF-7 cells bind to pS2 regulatory elements *in vitro* in a fashion that is very similar to binding *in vivo*. These results will help to identify proteins involved in regulation of the pS2 gene and provide a model system to define how estrogen-responsive genes are regulated.

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FOREWORD

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Larry N. Peltz 8-26-99

PI - Signature Date

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INTRODUCTION

Estrogen is a hormone of critical importance in the development and maintenance of normal reproductive tissues. Estrogen acts through an intracellular estrogen receptor (ER), which interacts with estrogen response elements (EREs) in target genes to initiate changes in transcription. One estrogen-responsive gene, which is expressed in many breast cancer cells, but not in normal mammary cells is the pS2 gene. The pS2 gene contains all of the elements of a classic hormone-responsive gene: an imperfect ERE (differs from the consensus ERE by a single nucleotide in the 3' half site), a TATA box, and a CAAT box. Our goal is to gain a better understanding of how estrogen-responsive genes are regulated in living cells. To do this we have utilized *in vivo* ligation mediated polymerase chain reaction footprinting to examine the regulatory elements of the pS2 gene in intact MCF-7 cells. To aid in the future identification of proteins bound to these elements, *in vitro* footprinting experiments were performed utilizing purified ER and nuclear extracts from estrogen-treated MCF-7cells.

BODY

We have addressed several of the Technical Objectives listed in the original grant. These objectives are listed below:

Task 1-2: DNase I treatment and LMPCR conditions that result in clear footprinting ladders have been optimized to examine the pS2 gene.

Task 3: K1 cells were treated with ethanol or 17β-estradiol, DNase I treated and genomic DNA isolated. *In vivo* footprints were detected on the noncoding strand in the regions of the TATA and CAAT boxes. We have designed a set of nested primers to examine the coding strand in this region. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the regions of the TATA and CAAT boxes.

Task 4: K1 cells were treated with trans-hydroxytamoxifen or ICI 182,780 and DNase I treated, but the regions of the TATA and CAAT boxes have not yet been examined.

Task 5-7: K1 cells were treated with ethanol, 17β-estradiol, trans-hydroxytamoxifen or ICI 182,780 and DNase I treated. Conditions have been optimized and *in vivo* footprints were detected with one set of nested primers designed to examine the noncoding strand of the pS2 ERE. We are in the process of optimizing conditions to examine the coding strand of the pS2 ERE using a second set of nested primers. In addition to these objectives stated in our original grant, we extended our investigations to examine dimethlysulfate *in vivo* footprints on the noncoding strand in the region of the pS2 ERE.

We have further extended our investigations to compare the time frame of pS2 mRNA expression to events occurring at the level of the gene. Northern blot analysis was utilized to

detect the relative levels of pS2 mRNA. The level of MCF-7 pS2 mRNA increased 16-fold after a 24 hours exposure to estrogen. These findings demonstrated that the pS2 gene was essentially inactive in the absence of estrogen, but responded robustly to estrogen treatment.

Examining the regulatory elements of the pS2 gene utilizing *in vivo* DNase I footprinting demonstrated that 24 hour estrogen treatment of MCF-7 cells resulted in extensive protection within and adjacent to the pS2 ERE. Interestingly treatment of MCF-7 cells with the antiestrogens 4-hydroxytamoxifen or ICI 182,780 each produced distinct protection patterns of the pS2 ERE. Also, the TATA and CAAT sequences were protected and flanked by hypersensitive sites in the presence and in the absence of hormone in ER-positive MCF-7 cells but not ER-negative MDA MB 231 cells. These findings indicate that the ER is involved not only in mediating the cellular response to hormone, but may also be involved in organization of the proximal promoter in the absence of hormone.

We have extended our studies to allow us to identify proteins interacting with the pS2 ERE. To determine whether proteins present in MCF-7nuclear extracts could bind to the pS2 ERE, gel mobility shift assays were carried out. When DNA fragments containing the pS2 ERE were combined with purified ER or nuclear extracts prepared from estrogen-treated MCF-7 cells, a major protein-DNA complex was formed. Since we anticipated that ER might bind to this region, antibodies to this proteins was included in separate binding reactions. The major protein-DNA complex was supershifted by the ER-specific antibody, H151, suggesting that ER bound efficiently to the pS2 ERE.

To aid in the future identification of proteins associated with the ERE bound ER, *in vitro* DNase I footprinting experiments were utilized, and the resulting protection patterns compared to

our *in vivo* footprinting experiments. DNA fragments, each containing the pS2 ERE, or the pS2 TATA box and CAAT box were incubated with increasing amounts of estrogen-treated MCF-7 nuclear extract or increasing amounts purified ER and exposed to DNase I. The resulting protection patterns were very similar to the protection patterns of our *in vivo* footprinting experiments. The pS2 perfect ERE half site was protected at the lowest protein concentrations followed by protection of the imperfect ERE half site at the highest protein concentrations. The CAAT box showed minimal protection with hypersensitive DNase I cleavage sites surrounding both the TATA and CAAT boxes. Because of the mirrored protection patterns in both our *in vitro* and *in vivo* footprinting experiments, it seems likely that proteins present in nuclear extracts from estrogen-treated MCF-7 cells bind to the pS2 regulatory elements in a fashion that is very similar to binding of proteins in intact MCF-7 cells. These results will help in identifying proteins involved in the transcriptional regulation of the pS2 gene and increase our understanding of estrogen-regulation genes.

APPENDIX

Key research accomplishments:

- *In vivo* DNase I footprinting experiments indicate the events occurring at the level of the pS2 gene are well coupled to the levels of pS2 mRNA in estrogen-treated MCF-7 human breast cancer cells.
- *In vivo* DNase I footprinting experiments demonstrate that the pS2 ERE is strongly protected after estrogen treatment and remains occupied for at least 24 hours after treatment.
- *In vivo* DNase I footprinting experiments demonstrate that antiestrogens mediate their effects by promoting the association of proteins with unique regions of the pS2 promoter.
- *In vivo* DNase I footprinting experiments demonstrate that the TATA and CAAT sequences are occupied and are flanked by hypersensitive sites in the presence and in the absence of estrogen-treatment.
- Purified ER and ER present in estrogen-treated MCF-7 cells can bind to the pS2 ERE, and is supershifted by an ER-specific antibody.
- *In vitro* DNase I footprinting experiments result in protection patterns that are very similar to the protection patterns seen in *in vivo* footprinting experiments.